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REC'D 05 AUG 2003

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This is to declare that in the Netherlands on June 13, 2002 under No. PCT/NL02/00389,
in the name of:

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an international patent application was filed for:

"Agonistic binding molecules to the human OX40 receptor",

and that the documents attached hereto correspond with the originally filed documents.

Rijswijk, July 15, 2003.

De Directeur van het Bureau voor de Industriële Eigendom,
voor deze,

Mw. I.W. Scheevelenbos-de Reus

Title: Binding molecules stimulating the human OX40 receptor.

Abstract

The present invention provides binding molecules that bind to and stimulate the human OX40 receptor. The invention also provides nucleic acids encoding such binding molecules. Methods for producing such binding molecules are also provided by the present invention. Said binding molecules and nucleic acids are useful in the stimulation of human T-cells, and can be used to enhance antigen-specific immune responses.

Title: Agonistic binding molecules to the human OX40 receptor.

Field of the invention

The present invention relates to the field of medicine, more in particular to binding molecules and nucleic acids useful in immunotherapy.

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Background of the invention

The OX40 receptor (OX40R) (also known as CD134, ACT-4, ACT35) is a member of the TNF receptor family and is expressed upon activation by CD4 T cells (WO 95/12673).

10 Triggering of this receptor via OX40L, present on activated B cells and dendritic cells enhances the proliferation of CD4 T cells during an immune response and influences the formation of CD4 memory T cells. Furthermore, the OX40R-OX40L system mediates adhesion of activated T cells to
15 endothelial cells, thus directing the activated CD4 T cells to the site of inflammation.

Inflammatory and autoimmune diseases, such as rheumatoid arthritis and inflammatory bowel disease, are characterized
20 by an infiltration of activated T cells at the site of inflammation, which is believed to orchestrate the response leading to chronic tissue destruction. In patients with inflammatory bowel disease OX40+ CD4 T cells can be found in the gut associated with sites of inflammation. In addition,
25 in patients suffering from acute graft-vs-host-disease elevated levels of OX40+ peripheral CD4 T cells are present in peripheral blood. In rheumatoid arthritis patients OX40+ CD4 T cells are present in synovial fluid while they are virtually absent from peripheral blood. Furthermore, OX40+
30 CD4 T cells are found in inflamed synovial tissue in addition to cells expressing the ligand for OX40R. This is in contrast to patients suffering from osteoarthritis, a

joint disease that is not mediated by inflammation, where both cell types could not be found in significant numbers. Thus in patients suffering from several inflammatory disorders elevated levels of OX40⁺ CD4 T cells are present at sites of inflammation, indicating that these cells may be involved in progression of autoimmune disease.

A blockade of the OX40R-OX40L pathway using antibodies or fusion proteins has led to the attenuation of disease progression in several animal models of autoimmune disease.

Besides their presence in autoimmune diseases, it has been shown that OX40⁺ T cells are present within tumor lesions containing tumor infiltrating lymphocytes and in tumor cell positive draining lymph nodes (Weinberg et al, 2000). It was shown in several tumor models in mice that engagement of the OX40R in vivo during tumor priming significantly delayed and prevented the appearance of tumors as compared to control treated mice (Weinberg et al, 2000). Hence it has been contemplated to enhance the immune response of a mammal to an antigen by engaging the OX40R by administering an OX40R binding agent (WO 99/42585; Weinberg et al, 2000). One possibility is to use a natural ligand of OX40R, i.e. OX40L, or fusion proteins thereof as an OX40R binding ligand. Such proteins however have a fixed affinity for the receptor that is not easily changed, may not have the circulatory retention time to exert the desired therapeutic effect, and may give rise to immunogenicity (Weinberg et al, 2000). Another possibility to stimulate T-cells by virtue of the OX40R pathway, is to use antibodies against this receptor (Kaleeba et al, 1998; Weinberg et al, 2000). A rat anti-mouse OX40R antibody named OX86 (Al-Shamkhani et al, 1996) appeared to engage OX40R in murine tumor models (Weinberg et al, 2000). However, to our knowledge agonistic antibodies that are capable of stimulating the human OX40R have not been disclosed in the art. Moreover, no human antibodies against the OX40R have been described for the treatment or

prevention-of tumours in humans. Therefore, a need exists for agonistic antibodies that stimulate the human OX40R, without the concomitant problems associated with the use of known antibodies.

Summary of the invention

The present invention discloses for the first time agonistic antibodies to the human OX40-receptor (OX40R). The invention provides agonistic binding molecules capable of binding to the human OX40 receptor, pharmaceutical compositions comprising the same, methods for obtaining these, and methods for producing the same in suitable host cells. In a preferred embodiment, said binding molecules are antibodies, antibody fragments or antibody conjugates. In one embodiment, said binding molecules are human binding molecules. It is another aspect of the present invention to provide nucleic acid molecules encoding at least the binding region of a binding molecule according to the invention. In particular embodiments, said nucleic acid is present in expressible format in a vector, or is present in a gene delivery vehicle. The invention further provides for the use of the binding molecules or nucleic acids according to the invention for enhancing the immune response in a human, for use in the treatment of the human or animal body, and for the preparation of a medicament to treat a human having or at risk of developing a disorder or disease. The invention also provides pharmaceutical compositions comprising binding molecules or nucleic acid molecules according to the invention.

Detailed description of the invention

In one aspect, the present invention provides an agonistic binding molecule capable of binding to the human OX40 receptor (OX40R). A binding molecule according to the

5 invention is agonistic when it stimulates T-cell proliferation or function in a costimulation assay, such as described in example 6 of the present invention. A binding molecule can be one molecule or comprise more molecules or subunits in the form of a complex. In one embodiment, a
10 binding molecule can comprise a natural ligand of the OX40R or a variant of the natural ligand still capable of binding to the human OX40R. In a preferred embodiment, a binding molecule according to the invention is an antibody, an antibody fragment, or an antibody conjugate. The term
15 antibody as used herein includes immunoglobulin isotypes and subclasses, such as IgG1, IgG2, IgG3, IgG4, IgA1, IgA2, IgM, IgE, and the like. It also includes engineered antibody forms such as chimeric antibodies, humanized antibodies, bispecific antibodies, and the like. Antibody fragments are
20 defined herein as binding molecules that comprise an antigen binding domain, and such fragments include F(ab'), F(ab')₂, scFv, Fv, dAb, Fd, diabodies, tetrabodies, single domain antibodies, and the like. An antibody conjugate comprises an antibody or antibody fragment, and another moiety. This
25 other moiety may be a radiolabel, a toxin, an enzyme, a non-enzymatic protein or non-enzymatic part of a protein, and the like. Such moieties may be conjugated chemically after synthesis of the antibody or fragment thereof.

Alternatively, other moieties comprising a protein or part
30 thereof may also be conjugated directly during synthesis, i.e. in the form of a fusion protein.

Typically, a binding molecule as used herein is defined as being able to bind to its target with an affinity constant (KD) that is lower than 0.2×10^{-4} M, more preferably lower
35 than 10^{-5} M, more preferably lower than 10^{-6} M, still more preferably 10^{-7} M, or less. Typically, monoclonal antibodies

may have affinity constants as low as 10^{-11} M, or even lower.

In one embodiment, the binding molecule according to the invention is a human binding molecule. A human antibody according to the invention lacks murine-derived sequences,

5 in contrast to mouse antibodies obtained by hybridoma technology (Kohler and Milstein, 1975), or variants thereof such as chimeric antibodies or humanized antibodies. Human antibodies have the advantage that when administered to humans an anti-antibody immunogenic response will be

10 extremely low or absent, whereas the murine derived antibodies can give rise to such responses quite extensively (Van Kroonenburgh and Pauwels, 1988). A human binding molecule is defined herein as derived from a human, based

upon a human sequence, or derived from or based upon a human sequence and subsequently modified. A binding molecule is

15 for instance based upon a human sequence when it has been obtained from a library of human binding molecules. Such a library may also comprise human binding molecules that are based upon a human sequence but containing mutations, e.g. a

20 semi-synthetic library, as was used to obtain molecules according to the present invention. Immunizing a mouse that comprises human immunoglobulin loci can also give rise to human antibodies. 'Derived from' as used herein, means that nucleic acid sequences, genes, or proteins that are normally

25 found in humans, are used for the generation or isolation of recombinant human binding molecules according to the invention. Methods to generate such recombinant human

binding molecules derived from humans may include general molecular biology methods such as cloning of genetic

30 information into desired constellations by use of restriction enzymes, and the like. 'Based upon' as used herein, is meant to include the synthetic construction of genetic information based upon knowledge of such genetic

information. Such methods include the use of human or human

35 derived genetic material as a template for PCR to construct a new binding molecule encoding construct that is based upon

the sequence of the template, the construction of completely synthetic genetic information with a desired sequence e.g. by linking synthetic oligonucleotides to a desired construct, and the like. It is to be understood that

5 'derived from' or 'based upon' does not exclusively mean a direct cloning of the wild type DNA. A person skilled in the art will also be aware of the possibilities of molecular biology to obtain mutant forms of a certain piece of nucleic acid. These mutations may render a different functionality,
10 but they may also be silent in a way that certain mutations do not alter the functionality of that particular piece of DNA and its encoded protein. A person skilled in the art will appreciate the fact that certain deletions, swaps, (point)mutations, additions, etc. may still result in a
15 nucleic acid that has a similar function as the original nucleic acid. It is therefore to be understood that such alterations that do not significantly alter the functionality of the encoded OX40R binding molecule are within the scope of the present invention. Human antibodies
20 according to the invention may therefore also contain (semi-)synthetic regions, e.g. in the CDR regions. It is for instance possible to alter the CDR regions of the variable domains of binding molecules by site-directed mutagenesis, oligo-directed mutagenesis, error-prone PCR, cloning of
25 restriction fragments, and the like (see e.g. US patent 5,225,539 for altering antibodies). It will be clear that the resulting binding molecules are still within the scope of the present invention.

Antibodies, antibody fragments, or antibody conjugates
30 according to the invention comprise an antigen binding domain. The antigen binding domain describes the part of an antibody which comprises the area which specifically binds to and is complementary to part or all of an antigen. Where an antigen is large, an antibody may only bind to a
35 particular part of the antigen, which part is termed an epitope. An antibody binding domain may be provided by one

or more antibody variable domains. Preferably, an antigen binding domain comprises an antibody light chain variable region (VL) and an antibody heavy chain variable region (VH). These regions comprise parts that are relatively conserved (the framework regions) and the hypervariable regions/complementarity determining regions (CDRs), which generally define much of the specificity of antibodies. In one embodiment, the present invention provides binding molecules according to the invention, wherein said binding molecules comprise a CDR3 region comprising an amino acid sequence chosen from DRYSQVHYALDY and YDNVMGLYWFDY, or a variant thereof. These CDR3 regions have been identified in the present application as being part of the agonistic binding molecules Sc02008 and Sc02023 (Table 1, Fig. 14). A variant is defined as a binding molecule comprising at least one mutation in the given (parent) CDR3 sequence, but still being capable of binding the human OX40R. Using the given sequences, variants can be constructed without undue effort by general methods of molecular biology, such as random mutagenesis, site-directed mutagenesis, oligonucleotide-directed mutagenesis, and the like, generally well known to the person skilled in the art of molecular biology. A nucleic acid encoding a variant is usually capable of hybridising to the nucleic acid molecules according to the invention under stringent conditions, known to the person skilled in the art. After creating such variants, it will also be easy to check whether the resulting variants still bind to the OX40R, and also whether they are agonistic binding molecules, e.g. using methods described in the present invention. It is therefore to be understood that such variants are included within the scope of the present invention. In one embodiment, such variants are capable of competing for the same binding site on OX40R as the binding molecule comprising the parent CDR3.

As discussed, it is possible for the person skilled in the art to mutate certain parts of the variable regions,

including but not limited to the CDR regions, to obtain variants of said variable region, which may differ in quantitative or qualitative aspects from the starting variable region. It is thus possible to obtain variants of the molecules of the present invention, binding to the OX40R with a different affinity, said affinity being either higher or lower than the OX40R binding molecules used as a starting point. The skilled person will be able to test the affinity and other characteristics of such variant binding molecules, and determine an optimal sequence, without undue effort. In general, a higher affinity is preferred, and preferably the affinity constant is in the nanomolar or subnanomolar range, e.g. 0.1-10 nM. The invention provides antibodies with an affinity constant (KD) that is lower than 10^{-6} M, more preferably lower than 10^{-7} M, even more preferably lower than 10^{-8} M, yet even more preferably lower than 10^{-9} M. In preferred embodiments the binding molecules or variants according to the invention have an affinity constant between 10^{-8} and 10^{-12} M. More preferably, said affinity is between 10^{-9} and 10^{-12} M. It is to be understood that the variants with different affinity are included in the scope of the present invention.

In another aspect, the present invention provides binding molecules according to the invention, wherein said binding molecules act synergistically in vitro with OX40L. An advantage of such binding molecules could be that they may enhance the effect of OX40L present in vivo, rather than only substituting it. Such synergistic activity can be determined by a functional assay described in example 6 of the present application.

It is another aspect of the present invention to provide a nucleic acid molecule encoding at least the binding region of a binding molecule according to the invention.

Such nucleic acid molecules can be used as intermediates for cloning purposes, e.g. in the process of affinity maturation

described above.

The nucleic acid sequences according to the present invention are also useful for the production of the binding molecules according to the invention, e.g. by providing
5 cells capable of expressing proteins with expression cassettes comprising said nucleic acids. In one embodiment, the binding molecule according to the invention is obtained by expressing a nucleic acid molecule according to the invention in suitable host cells. In another embodiment a
10 nucleic acid sequence according to the invention is provided wherein said nucleic acid is present in expressible format in a vector. It is yet another aspect of the present invention to provide a method for producing a binding molecule according to the invention, said method comprising
15 the steps of: i) introducing into a suitable host cell a nucleic acid sequence or sequences encoding said binding molecule, or a precursor thereof, operably linked to a sequence or sequences capable of driving expression of said sequence or sequences encoding said binding molecule, or a
20 precursor thereof, in said cell; ii) culturing said cell under conditions conducive to the production of said binding molecule. Said binding molecule produced by said cell can be further isolated and/or purified using methods known to the person skilled in the art. Expression cassettes are a form
25 wherein a nucleic acid is present in expressible format. These are obtained by constructing a suitable regulatory sequence in operable association with the genetic information encoding the protein of interest, in this case the OX40R binding molecule. The nucleic acids according to the invention in operable association with a transcriptional
30 regulatory element, can be cloned into a vector for convenience. A 'vector' as used herein is a DNA molecule, capable of replication in a host organism, into which genetic information is inserted to construct a recombinant
35 DNA molecule. The term vector is meant to include plasmids, phagemids, cosmids, yeast artificial chromosomes, and the

like. Non-limiting examples wherein the sequence encoding an antibody or antibody fragment binding to and stimulating OX40R is regulated by a AOX1 promoter or a CMV promoter are given in examples 5 and 9 of the present application.

- 5 Vectors useful for constructing various antibody formats from scFv sequences obtained by the phage display methods have been described (Boel et al, 2000). Examples of vectors comprising expression cassettes containing CMV promoters that regulate expression of antibodies in eukaryotic cells
- 10 can e.g. be found in WO 00/63403. Introduction of such an expression cassette into a suitable expression system can be done according to a variety of methods known to the person skilled in the art, including but not limited to electroporation, transfection using lipofectamin or other
- 15 transfecting agents, virus infection, calciumphosphate transfection, and the like. A suitable host cell is any cell that is capable of producing protein, including bacteria, yeast, or higher eukaryotic cells. In general the expression cassette used should be compatible with the host cell used.
- 20 In one embodiment, the host cell is a mammalian cell, such as a Chinese hamster ovary (CHO) cell, a BHK cell, and the like. Mammalian cells provide expressed proteins with posttranslational modifications that are most similar to natural molecules of mammalian origin. Since the present
- 25 invention deals with molecules binding to a human protein, and such binding molecules may have to be administered to humans, a completely human expression system would be preferred. Therefore, even more preferably, said host cell is a human cell, such a 293 or PER.C6TM. In preferred
- 30 embodiments, the producing human cells comprise at least a functional part of a nucleic acid sequence encoding an adenovirus E1 region in expressible format. In a most preferred embodiment, said host cell is derived from a human retina and immortalized with nucleic acid comprising
- 35 adenoviral E1 sequences, such as a PER.C6TM cell and derivatives thereof. Production of recombinant proteins in

host cells-can be done according to procedures well known to the person skilled in the art. The use of the PER.C6™ cell line as a production platform for proteins of interest has been described in WO 00/63403.

5

Another aspect of nucleic acids according to the present invention, is their potential for use in gene-therapy or vaccination applications. Therefore, in another embodiment of the invention, nucleic acids according to the invention
10 are provided wherein said nucleic acid is present in a gene delivery vehicle. A 'gene delivery vehicle' as used herein refers to an entity that can be used to introduce nucleic acid into cells, and includes liposomes, recombinant viruses, and the like. In specific embodiments, nucleic acid
15 molecules according to the invention are incorporated into an adenovirus, a retrovirus, or into another virus. Such applications of the nucleic acid sequences according to the invention are included in the present invention. The person skilled in the art will be aware of the possibilities of
20 recombinant viruses for administering sequences of interest to cells. The administration of the nucleic acids of the invention to cells can result in an enhanced immune response.

Agonistic binding molecules and nucleic acids of the
25 invention are capable of engaging the OX40R in vivo, and thereby enhance antitumor activity. Methods to administer the binding molecules or nucleic acids are described in WO 99/42585. Such application of the nucleic acids or binding molecules according to the invention is referred to as
30 immunotherapy. All kinds of tumours are potentially amenable by treatment according to this approach, including but not limited to carcinoma of the breast, pancreas, lung, kidney, ovary, colon and bladder, as well as sarcomas, melanomas, and tumours of the hemopoietic system.

35 It is therefore another aspect of the present invention to use the binding molecules or the nucleic acids according to

the invention for enhancing the immune response in a human or animal. In a particular embodiment, said immune response is directed to a tumour antigen. In yet another embodiment, the invention provides a binding molecule or a nucleic acid according to the invention for use in the treatment of the human or animal body. In yet another aspect the present invention provides the use of the binding molecules and/or the nucleic acids according to the invention for the preparation of a medicament for treating a human having or at risk of developing a disease or disorder. According to a particular embodiment, said disease or disorder is cancer.

It is another aspect of the present invention to provide a method for obtaining a binding molecule or nucleic acid according to the invention, said method comprising the steps of i) bringing a phage library of binding molecules into contact with at least part of the human OX40R under conditions that allow for binding, ii) at least one step of selecting a phage binding to said at least part of the human OX40R, and iii) isolating said binding molecule or nucleic acid from a phage binding to said at least part of the human OX40R. Phage display methods for obtaining antibodies are known to the person skilled in the art, and are e.g. described in (US patent 5,696,108; Burton and Barbas, 1994; de Kruif et al., 1995). Generally, one or more selection rounds are required to separate the phages of interest from the large excess of non-binding phages. The phage display method can be extended and improved by subtracting non-relevant binders during screening by addition of an excess of non-target molecules that are similar but not identical to the target, and thereby strongly enhance the chance of finding relevant binding molecules (US patent 6,265,150). Applicant shows that OX40R binding molecules can be obtained by a method according to the invention with at least one selection step in the presence of human tissue or cell material comprising OX40R (see example 3 of the present

application). In this method, subtraction can be done by the presence of T-cells and other lymphocytes that do not express OX40R. Alternatively, binding molecules may be obtained by a method according to the invention comprising at least a selection step in the presence of at least part of the human OX40R, e.g. isolated OX40R, the extracellular part thereof, fusion proteins comprising such, and the like.

It is another aspect of the present invention to provide pharmaceutical compositions comprising binding molecules or nucleic acid molecules according to the invention, and a pharmaceutically acceptable carrier. Pharmaceutically acceptable carriers as used herein are exemplified, but not limited to, adjuvants, solid carriers, water, buffers, or other carriers used in the art to hold therapeutic components, or combinations thereof.

EXAMPLES

To illustrate the invention, the following examples are provided, not intended to limit the scope of the invention.

Example 1.

Selection of phage carrying single chain Fv fragments specifically recognizing human OX40R using OX40Ig fusion protein.

Antibody fragments were selected using antibody phage display libraries and MAbstractTM technology, essentially as described in US patent 6,265,150 and in WO 98/15833. All procedures were performed at room temperature unless stated otherwise. A human OX40-Ig fusion protein consisting of the extracellular domain of human OX40R linked to the CH2 and CH3 domains of human IgG1 was obtained commercially (Alexis Biochemicals) and coated for 2 hours at 37° onto the surface of MaxisorpTM plastic tubes (Nunc) at a concentration of 1.25 µg/ml. The tubes were blocked for 1 h in 2% fat free milk powder dissolved in PBS (MPBS). Simultaneously, 500 µl (approximately 10¹³ cfu) of a phage display library expressing single chain Fv fragments (scFv's) essentially prepared as described by De Kruif et al. (1995a) and references therein, was added to two volumes of 4% MPBS. In addition, human serum was added to a final concentration of 15% and blocking was allowed to proceed for 30-60 min. The OX40-Ig-coated tubes were emptied and the blocked phage library was added. The tube was sealed and rotated slowly for 1 h, followed by 2 h of incubation without rotation. The tubes were emptied and washed 10 times in PBS containing 0.1% Tween-20, followed by washing 5 times in PBS. 1 ml glycine-HCl, 0.05 M, pH 2.2 was added, and the tube was rotated slowly for 10' min. The eluted phages were added to 500 µl 1M Tris-HCl pH 7.4. To this mixture, 3.5 ml of

exponentially growing XL-1 blue bacterial culture was added. The tubes were incubated for 30 min at 37°C without shaking. Then, the bacteria were plated on 2TY agar plates containing ampicillin, tetracycline and glucose. After overnight
5 incubation of the plates at 37°C, the colonies were scraped from the plates and used to prepare an enriched phage library, essentially as described by De Kruif et al. (1995a). Briefly, scraped bacteria were used to inoculate 2TY medium containing ampicillin, tetracycline and glucose
10 and grown at a temperature of 37°C to an OD_{600nm} of ~0.3. Helper phages were added and allowed to infect the bacteria after which the medium was changed to 2TY containing ampicillin, tetracycline and kanamycin. Incubation was continued overnight at 30°C. The next day, the bacteria were
15 removed from the 2TY medium by centrifugation after which the phages were precipitated using polyethylene glycol 6000/NaCl. Finally, the phages were dissolved in a small volume of PBS-1% BSA, filter-sterilized and used for a next round of selection. The selection/re-infection procedure was
20 performed twice. After the second round of selection, individual *E.coli* colonies were used to prepare monoclonal phage antibodies. Essentially, individual colonies were grown to log-phase and infected with helper phages after which phage antibody production was allowed to proceed
25 overnight. Phage antibody containing supernatants were tested in ELISA for binding activity to human OX40-Ig coated 96 wells plates.

30 Example 2.

Validation of the human OX40R-specific scFv's.

Selected phage antibodies that were obtained in the screen described above, were validated in ELISA for specificity. For this purpose, human OX40-Ig was coated to
35 Maxisorp ELISA plates. After coating, the plates were blocked in 2% MPBS. The selected phage antibodies were

incubated in an equal volume of 4% MPBS. The plates are emptied, washed once in PBS, after which the blocked phages were added. Incubation was allowed to proceed for 1 h, the plates were washed in PBS 0.1% Tween-20 and bound phages
5 were detected using an anti-M13 antibody conjugated to peroxidase. As a control, the procedure was performed simultaneously using a control phage antibody directed against thyroglobulin (De Kruif et al. 1995a and 1995b), which served as a negative control. As shown in Figure 1,
10 several phage antibodies displayed significant binding to the immobilized OX40-Ig fusion protein.

The phage antibodies that bound to human OX40-Ig were subsequently tested for binding to human serum IgG to exclude the possibility that they recognized the Fc part of
15 the fusion protein. None of the selected anti-OX40R phages demonstrated binding to human IgG.

In another assay the phage antibodies were analyzed for their ability to bind PER.C6TM cells that express human OX40R. To this purpose PER.C6TM cells were transfected with
20 a plasmid carrying a cDNA sequence encoding human OX40R or with the empty vector and stable transfectants were selected using standard techniques known to a person skilled in the art (Coligan et al., 2001). For flow cytometry analysis, phage antibodies were first blocked in an equal volume of 4%
25 MPBS for 15 min at 4°C prior to the staining of the OX40R- and control transfected PER.C6TM cells. The blocked phages were added to a mixture of unlabelled control transfected PER.C6TM cells and OX40R transfected PER.C6TM cells that were labelled green using a lipophylic dye (PKH67, Sigma). The
30 binding of the phage antibodies to the cells was visualized using a biotinylated anti-M13 antibody (Santa Cruz Biotechnology) followed by streptavidin-phycoerythrin (Caltag). As shown in Figure 2, the selected anti-human OX40R phage antibodies SC02008, SC02009, SC02010, SC02011, SC02012 and SC02021 selectively stained the PER.C6TM OX40R
35

transfectant while they did not bind the control transfectant.

In another assay the phage antibodies were analyzed for their ability to bind to OX40R positive CD4+

5 from synovial fluid from RA-patients. As a control the staining pattern of the anti-OX40R phage antibodies on peripheral blood mononuclear cells (MNC) is also shown.

Inflamed tonsils were obtained from patients undergoing routine tonsillectomy. Tonsils were minced and the MNC

10 fraction was isolated by density centrifugation. Flow cytometric analysis of the binding of the anti-OX40R phage antibodies to the OX40+ CD4+ T cells was performed as

described above. The CD4+ T cells were distinguished from total tonsil MNC using a FITC conjugated antibody against

15 CD4. As shown in Figures 3A and 3B the selected anti-human OX40R phage antibodies SC02008, SC02009, SC02010, SC02011, SC02012 and SC02021 selectively stain a subset of CD4+ T

cells within tonsil and synovial fluid mononuclear cells respectively, while they display minor staining of

20 peripheral blood CD4+ T cells (Figure 3C).

25 Example 3.

Selection of phage carrying single chain Fv fragments specifically recognizing human OX40R using OX40⁺ CD4⁺ T cells.

30 Phage selection experiments were performed as described supra, using lymphocytes as target. An aliquot of the phage library (500 μ l, approximately 10^{13} cfu) were blocked with 2 ml RPMI/10%FCS/1%NHS for 15' at RT. Tonsil MNC ($\sim 10 \times 10^6$ cells) were added to the blocked phage-library and incubated

35 for 2,5 hr while slowly rotating at 4°C. Subsequently, the

cells were washed twice and were resuspended in 500µl RPMI/10%FCS and incubated with a FITC-conjugated anti-CD4 antibody (Bectin Dickinson) and a phycoerythrin-conjugated anti-OX40R antibody (Becton Dickinson) for 15' on ice. The cells were washed once and transferred to a 4 ml tube. Cell sorting was performed on a FACSvantage fluorescence-activated cell sorter (Becton Dickinson), ~15.000 CD4+OX40+ cells were sorted. The sorted cells were spun down, the supernatant was saved and the bound phages were eluted from the cells by resuspending the cells in 500µl 50mM Glycin pH2.2 followed by incubation for 5 min. at room temperature. The mixture was neutralized with 250µl 1M Tris-HCl pH 7.4 and added to the rescued supernatant. Collectively these phages were used to prepare an enriched phage library as described earlier. The selection/re-infection procedure was performed twice. After the second round of selection, monoclonal phage antibodies were prepared and tested for binding to tonsillar OX40+ CD4+ T cells. Selected phage antibodies that met this criterium were subsequently tested for binding to OX40R-transfected PER.C6TM cells. The results in Figure 4 show that the selected phages SC02022 and SC02023 selectively bind to a subset of CD4+ T cells within tonsil mononuclear cells (Fig. 4A) and that they bind to the human OX40R PER.C6TM transfectant (Fig. 4B).

25

Example 4.**Characterization of the human OX40R-specific scFv's.**

From the selected OX40R-specific scFv clones plasmid DNA was obtained and nucleotide sequences were determined according to standard techniques. Nucleotide sequences and amino acid translations of scFv's SC02008, SC02009, SC02010, SC02011, SC02012, SC02021, SC02022 and SC02023 are shown in figures 5-12. The VH and VL gene identity and HCDR3 compositions of the anti-human OX40R scFv's are depicted in table 1. The scFv clones SC02008, SC02010, SC02011 and

SC02023 were deposited at the ECACC under deposit numbers 02051563, 02051560, 02051561 and 02051562 respectively.

5 **Example 5.**

Production of human OX40R specific bivalent scFv in *Pichia Pastoris*.

10 Methods for the cloning and expression of scFv fragments in the *Pichia pastoris* system were based on protocols provided by the manufacturer (Invitrogen) in "A Manual of Methods for Expression of Recombinant Proteins Using pPICZ and pPICZ α in *Pichia pastoris* (Version F)".

15 The bivalent scFv expression vector pPicZbiFVH was constructed from the vector pPICZ α B (Invitrogen) following standard molecular biology techniques known to a person skilled in the art (see Figures 13A and 13B). Three modifications were introduced in the pPICZ α B:

20

1. A restriction site (NcoI) was introduced by PCR-generated point mutation directly after the KEK2 cleavage site of the signal peptide to facilitate cloning into the vector.
- 25 2. A second NcoI restriction site was removed by PCR generated point mutation inside the coding region of the sh ble gene.
3. A synthetic fragment comprising the hinge region of murine IgG3 and a linker fragment was introduced
- 30 between the restriction sites NotI and XbaI.

All modifications were confirmed by sequencing. ScFv's were cloned into pPicZbiFVH from the phage display expression vector by directional cloning using the restriction sites

35 NcoI and NotI. The *Pichia pastoris* strain SMD1168 kek1:suc1

(ATCC # 204414) was transformed with 5-10 µg of linearized construct cDNA by electroporation according to the manufacturer's protocols (supra). The transformed cells were plated on YPDS agar containing 250 µg/ml Zeocin and

5 incubated at 30°C for 3-4 days.

High producing clones were selected by colony lift immunoblot screening, as follows. Pre-wet nitrocellulose membranes were layered over the transformation plates and a fraction of each colony was lifted onto the membrane. The

10 membrane was then placed colony side up on YPD agar

containing 0.5% methanol and incubated overnight at 30°C.

The membranes were then washed repeatedly with Tris buffered saline containing 0.5% Tween 20 (TBST) to removed colonies then blocked for 30 min with TBST + 4% non-fat milk powder.

15 The membranes were then placed in TBST + 4% non-fat milk powder + horseradish peroxidase conjugated anti-c-myc antibody (Roche) for 1 hr. Finally the membranes were washed extensively in TBST followed by a PBS washing step and scFv-secreting colonies were revealed by a chemofluorescence

20 detection system (Apbiochem). Selected high producers were purified by streaking on YPD plates and were subsequently used for bivalent scFv expression. Small-scale expression cultures were carried out in shaker flasks essentially as described by the manufacturer's protocols (supra). BMGY

25 medium was used for the cell expansion phase while BMMY medium was used during the bivalent scFv expression phase.

After 48hr of induction supernatants were clarified by repeated centrifugation. The supernatant was conditioned for purification by the addition of 1M Na₂HPO₄ pH 8 to a

30 concentration of 20 mM, 0.5M Imidazole to a concentration of 10 mM, 5M NaCl to a concentration of 500 mM. Hereafter, the samples were purified by immobilized metal affinity

chromatography followed by anion exchange chromatography on an AKTAprime FPLC system (Pharmacia). A 5ml HiTrap chelating

35 column (Pharmacia) was charged with NiSO₄ and equilibrated

according to manufacturers instructions. Conditioned supernatant was loaded directly on to the column and washed extensively in equilibration buffer (20mM Na₂PO₄ pH8, 10mM Imidazole). Bivalent scFv were eluted directly off the column on to a 1ml sepharose Q HP column (Pharmacia) in the presence of 250mM Imidazole pH8.5. The column was then washed in 20mM Tris-Cl pH8, then briefly in 20mM Na₂PO₄ pH 7.3, and bivalent scFv's were eluted off the column over a gradient of 0-0.5M NaCl in 7 column volumes. Fractions were then measured for protein content and were analyzed for activity and purity.

Example 6.

Functional analysis of bivalent scFv specifically recognizing human OX40R

The anti-OX40R bivalent scFv's were validated for their ability to bind to OX40+ CD4+ T cells within tonsil MNC. Tonsil MNC samples were obtained as described supra and were stained with bivalent scFv's at a concentration of 5 µg/ml at 4°C. Binding of the bivalent scFv's was visualized using a biotinylated anti-myc antibody (9E10, Santa Cruz Biotechnology) followed by streptavidin-phycoerythrin (Caltag). The bivalent anti-OX40R scFv's displayed a similar staining pattern as compared to the same scFv's when analyzed in phage antibody format.

The anti-OX40R bivalent scFv's were analyzed for their ability to interfere with OX40R-mediated signaling in a costimulation assay. To this purpose 293T cells transfected with either the empty vector or with an OX40L cDNA-containing plasmid (pCDNA3.1zeo(+), InVitrogen) using the lipofectamine reagent according to standard protocols. 48 hours after transfection, the cells were harvested, paraformaldehyde fixed and analyzed for cell surface expression of OX40L by flow cytometry (OX40L was visualized

using the OX40-Ig fusion protein followed by incubation with a biotinylated goat-anti-human Fc polyclonal antibody (Caltag) and streptavidin-phycoerythrin (Caltag)). To cocultures of 1.5×10^3 293T transfectants and 4×10^5 T cells, which were activated with a submitogenic dose of 50 ng/ml of PHA (Abbot Murex), several concentrations of the bivalent anti-OX40R or control scFv's were added. T cells were purified via negative selection using the MACS system and a pan-T cell isolation kit (Myltenyi Biotec) from PBMC that were obtained from healthy donors by Ficoll-Hypaque density gradients. The cultures were performed in U bottom 96 well plates for 5 days and the proliferation of the T cells was measured by ^3H -thymidine incorporation during the last 16 hours of culture. As shown in figures 14A and 14B, scFv's SC02008 and SC02023 display agonistic (stimulating) function in that they induce T cell proliferation in a concentration dependent manner when incubated with the mock-transfected 293T cells. Interestingly, these agonistic anti-OX40R scFv's demonstrate a synergistic stimulatory effect when co-incubated with the OX40L-transfected 293T cells as compared to the level of proliferation that is reached with the same transfectant in the presence of a control bivalent scFv antibody.

In a similar costimulation assay 4×10^5 purified T cells that had been labelled with CFDA-SE (Molecular Probes) were incubated with 1.5×10^3 mock-transfected 293T T cells under similar conditions as described above. At day 4 and day 5 of culture samples were obtained, stained with APC-conjugated anti-CD4 antibody (Pharmingen) and with phycoerythrin-conjugated anti-OX40R antibody (Becton Dickinson) and were analyzed by flow cytometry. This experiment demonstrates the agonistic function of scFv's SC02008 and SC02023 by showing that they specifically induce T cell proliferation in OX40+ CD4+ T cells as shown by the diminished intensity of the CFDA-SE signal, which is indicative for the proliferation of these cells.

In another costimulation assay 4×10^5 purified T cells are incubated with a combination of 2 $\mu\text{g/ml}$ of anti-CD3 antibody (OKT3) and several concentrations of the bivalent anti-OX40R or control scFv's. The cultures are performed in U bottom 96 well plates for 5 days and the proliferation of the T cells is measured by ^3H -thymidine incorporation during the last 16 hours of culture.

In another costimulation assay the experiments described above are performed using a well characterized human CD4+ T cell clone that homogeneously expresses OX40R. In addition to proliferation, OX40R-mediated stimulation of the CD4+ T cells is assessed by analyzing the supernatants of the T cell cultures for the presence of the cytokines IL-2, IFN γ and TNF α . Furthermore, in these experiments the OX40R-dependence of the induced stimulation is demonstrated by performing the assays in the presence of various concentrations of OX40-Ig fusion protein or a control human IgG1 antibody.

Example 7. Immunohistochemistry

The anti-OX40R bivalent scFv's are analysed for their ability to bind to OX40 $^+$ cells in inflamed tonsil and tumor sections with infiltrating lymphocytes. To this purpose, frozen sections of inflamed tonsil and breast tumor are cut, mounted on glass slides and are dried at room temperature. The sections are blocked with PBS containing 4% BSA and 10% normal human serum and incubated with the bivalent anti-human OX40R scFv's for 45 minutes at room temperature. To detect bound scFv fragments the sections are incubated with the 9E10 anti-myc antibody (Roche) followed by an anti-mouse IgG envision reagent (DAKO). The sections are counterstained with hematoxinilin to visualize nucleated cells within the sections.

**Example 8. In vivo analysis of enhanced immune response
induced by agonistic OX40R binding molecules.**

To determine the cross-reactivity of the anti-human OX40R antibodies with mouse OX40R, splenic OX40+ CD4+ T cells are analyzed by flow cytometry. Murine OX40+ T cells are generated by stimulating C57Bl6 splenic CD4 T cells that are isolated using an anti-CD4-phycoerythrin antibody (PharMingen) and anti-phycoerythrin labeled MACS beads (Myltenyi Biotec) with a mitogenic dose of PHA and IL2. The cells are analyzed after 72 hours of stimulation with a rat antibody against mouse OX40R and with the panel of bivalent anti-human OX40R scFv's as described supra. In case the agonistic anti-human OX40R bivalent sFv's display cross reactivity with mouse OX40R, the OX40R receptor can be engaged in vivo with these agonistic antibodies in a bivalent scFv format or as an IgG molecule to demonstrate the delivery of a costimulatory signal to effector T cells. To demonstrate the effect of providing an agonistic OX40R binding molecule to T cells during tumor priming in vivo, a MCA 303 sarcoma tumor model in C57BL/6 mice is used as described by Weinberg et al (2000) and in WO 99/42585. Mice are inoculated subcutaneously at day 0 with $1-3 \times 10^5$ MCA 303 sarcoma tumor cells. Three days later the animals are given intraperitoneal injections with the agonistic anti-human OX40R binding molecule at doses ranging from 100-500 μ g per animal. A second dose is given 7 days after tumor inoculation. The animals are then monitored for tumor growth for over 50 days, animals are sacrificed when tumor sizes exceed 1 cubic cm. When animals that are given the agonistic anti-human OX40R reagent remain tumor free (or have tumours smaller in size than control animals) while animals that are given the tumor cells alone have to be

sacrificed, this then indicates that engagement of the OX40R by the agonistic anti-human OX40R reagent costimulates effector T cells to exert their tumor eradicating function. The experiment described above can also be performed in a transgenic mouse model in which human OX40R is expressed under a T cell specific promoter. Such a mouse can be created according to protocols known to the person skilled in the art of transgenic mouse models.

10

Example 9 Construction of fully human immunoglobulin molecules from the selected agonistic anti-human OX40R single chain Fv fragments.

To use the identified agonistic antibody fragments that recognize human OX40R for enhancing the immune response in a human it is desirable to generate human immunoglobulin molecules. The engineering and production of the human IgG4 monoclonal antibodies is essentially performed as described in detail by Boel et al. (2000). The VH and VL regions encoding the scFv anti-human OX40R antibodies are excised and recloned into vectors for expression of complete human IgG4 molecules in mammalian cells in a two-step cloning procedure. The scFv fragments are first cloned in pLEADER (Boel et al. 2000) to add the T cell receptor α chain HAVT20 leader peptide sequence and a splice donor site. In the second cloning step, the scFv containing the leader sequence and donor splice site are subcloned in pNUT-C γ 1 or pNUT-C κ expression vectors for the generation of the heavy and light chain, respectively as described by Boel et al. (2000). The pNUT expression plasmids can be used for recombinant expression in mammalian cells. Alternatively, vectors can be prepared that harbor both heavy and light chain in IgG format. The cloning procedures that are executed are essentially as described in WO 00/63403, using several

common cloning techniques very well known to persons skilled in the art of molecular biology.

The expression construct encoding the human IgG4 directed against human OX40R is stably integrated into
5 PER.C6TM cells and positive producer cells are selected for their resistance to G418 and for their production rate of recombinant IgG4 using ELISA and procedures known to persons skilled in the art (see WO 00/63403). Subsequently, the antibodies are purified over size-exclusion columns and
10 protein-A columns using standard purification methods used generally for immunoglobulins. The purified IgG4 antibodies are used in specificity experiments and in functional experiments, such as the costimulation assays described
supra.

Brief description of the figures**Figure 1**

Binding of anti-human OX40R phage antibodies that were
5 selected using OX40-Ig fusion protein to immobilized OX40-Ig
fusion protein

Figure 2

Binding of anti-human OX40R phage antibodies that were
10 selected using OX40-Ig fusion protein to human OX40R-
transfected PER.C6™ cells

Figure 3

Binding of anti-human OX40R phage antibodies that were
15 selected using OX40-Ig fusion protein to OX40+ CD4+ T cells

Figure 4

Binding of anti-human OX40R phage antibodies that were
selected using OX40+ CD4+ T cells to OX40+ CD4+ T cells and
20 to human OX40R-transfected PER.C6™ cells

Figure 5

Nucleotide sequence and amino acid translation of scFv
SC02008
25

Figure 6

Nucleotide sequence and amino acid translation of scFv
SC02009

Figure 7

Nucleotide sequence and amino acid translation of scFv
SC02010

Figure 8

35 Nucleotide sequence and amino acid translation of scFv
SC02011

Figure 9

Nucleotide sequence and amino acid translation of scFv
SC02012

5

Figure 10

Nucleotide sequence and amino acid translation of scFv
SC02021

10 **Figure 11**

Nucleotide sequence and amino acid translation of scFv
SC02022

Figure 12

15 Nucleotide sequence and amino acid translation of scFv
SC02023

Figure 13

20 Construction of the bivalent scFv expression vector
pPICZbiFVH

Figure 14

25 Functional activity of anti-human OX40R bivalent scFv's
SC02008 and SC02023 in an *in vitro* T cell costimulation
assay.

Table 1

5

	<u>scFv</u>	<u>HCDR3</u>	<u>V_H-germline</u>	<u>V_L-germline</u>
	Sc02008	DRYSQVHYALDY	V _H 3 DP47	V _K II
10	Sc02009	DRYVNTSNAFDY	V _H 3 DP29	V _K II
	Sc02010	DMSGFHEFDY	V _H 3 DP46/49/50	V _K I
	Sc02011	DRYFRQQNAFDY	V _H 3 DP42/47	V _K II
	Sc02012	ARAAGTIFDY	V _H 3	V _K I
	Sc02021	DRYITLPNALDY	V _H 3 DP46/49/50	V _K II
15	Sc02022	YDEPLTIYWFDS	V _H 3 DP44/45	V _K III
	Sc02023	YDNVMGLYWFDY	V _H 3 DP44/45	V _K III

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Claims

1. An agonistic binding molecule capable of binding to and stimulating the human OX40 receptor (OX40R).

2. A binding molecule according to claim 1, wherein said binding molecule is an antibody, an antibody fragment, or an antibody conjugate.

3. A binding molecule according to claim 1 or 2, wherein said binding molecule is a human binding molecule.

10

4. A binding molecule according to any one of claims 1-3, wherein said molecule comprises a CDR3 region comprising an amino acid sequence chosen from a) DRYSQVHYALDY and b) YDNVMGLYWFDY, or a variant thereof.

15

5. A binding molecule comprising a variant CDR3 region according to claim 4, wherein said variant is capable of competing for the same binding site as the binding molecule comprising the parent CDR3.

20

6. A binding molecule according to any one of claims 1-5, wherein said binding molecule acts synergistically in vitro with OX40 ligand (OX40L).

25 7. A nucleic acid encoding at least the binding region of a binding molecule according to any one of claims 1-6.

8. A nucleic acid according to claim 7, wherein said nucleic acid is present in expressible format in a vector.

30

9. A nucleic acid according to claim 7, wherein said nucleic acid is present in a gene delivery vehicle.

10. A method for obtaining a binding molecule or nucleic acid according to any one of claims 1-9, said method comprising the steps of

- 5 i) bringing a phage library of binding molecules into contact with at least part of the human OX40R under conditions that allow for binding,
- ii) at least one step of selecting a phage binding to said at least part of the human OX40R, and
- 10 iii) isolating said binding molecule or nucleic acid from a phage binding to said at least part of the human OX40R.

11. A method according to claim 10, wherein the at least part of the human OX40 receptor is present in the form of human cell material comprising the OX40 receptor or as
15 isolated molecules.

12. A pharmaceutical composition comprising a binding molecule according to any one of claims 1-6 or a nucleic acid according to any one of claims 7-9, and a
20 pharmaceutically acceptable carrier.

13. Use of a binding molecule according to any one of claims 1-6, or a nucleic acid according to any one of claims 7-9, or a pharmaceutical composition according to claim 12,
25 for enhancing the immune response in a human or animal.

14. Use according to claim 13, wherein said immune response is directed to a tumour antigen.

30 15. Use of a binding molecule according to any one of claims 1-6, or a nucleic acid according to any one of claims 7-9, or a pharmaceutical composition according to claim 12, for treating or preventing disease in a human or animal subject.

16. Use of a binding molecule according to any one of claims 1-6, or a nucleic acid according to any one of claims 7-9, or a pharmaceutical composition according to claim 12, for stimulating T-cells.

5

17. A binding molecule according to any one of claims 1-6, or a nucleic acid according to any one of claims 7-9, for use in the treatment of the human or animal body.

10 18. Use of a binding molecule according to any one of claims 1-6, or a nucleic acid according to any one of claims 7-9, for the preparation of a medicament to treat a human having or at risk of developing a disease or disorder.

15 19. Use according to claim 18, wherein said disease or disorder is cancer.

20. A method for producing a binding molecule according to any one of claims 1-6, comprising the steps of:

20 a) introducing into a suitable host cell a nucleic acid sequence or sequences encoding said binding molecule, or a precursor thereof, operably linked to a sequence or sequences capable of driving expression of said sequence or sequences encoding said binding molecule, or a precursor thereof, in said cell;

25 b) culturing said cell under conditions conducive to the production of said binding molecule.

21. A method according to claim 18, further comprising:

30 c) isolating and/or purifying said binding molecule.

22. A method according to claim 18 or 19, wherein said cell is of human origin.

35 23. A binding molecule according to any one of claims 1-6, wherein said binding molecule is obtained by expressing a

nucleic acid molecule according to claim 8 in suitable host cells.

24. A binding molecule according to claim 22, wherein
5 said suitable host cells are of human origin.

Figure 1

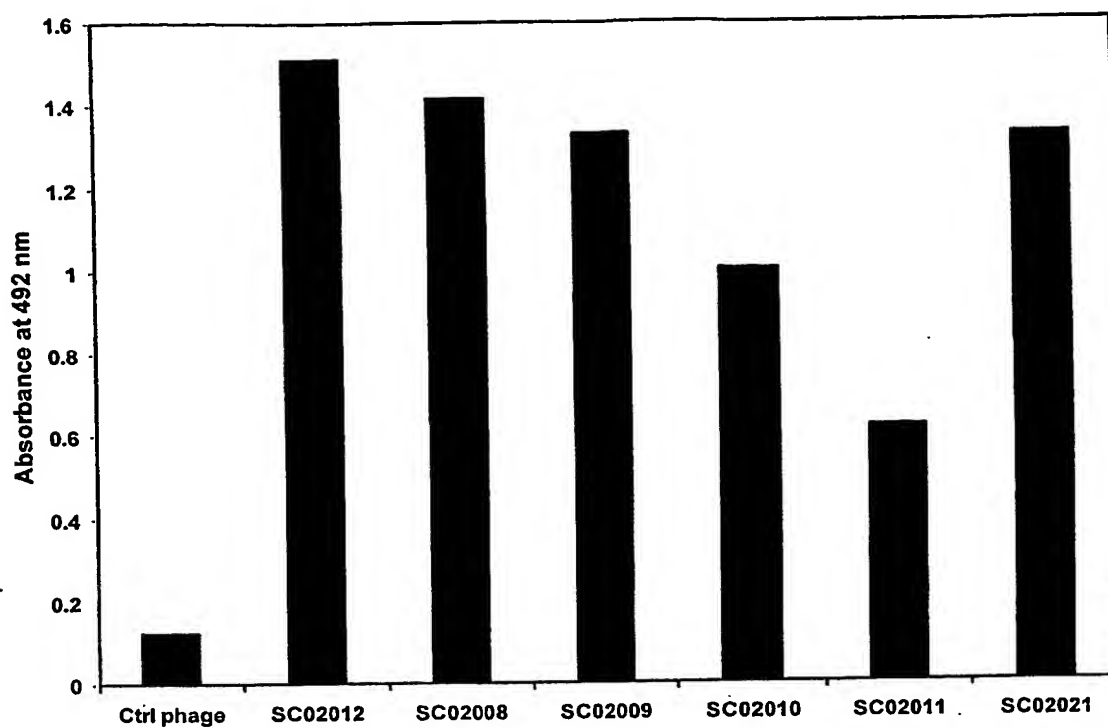
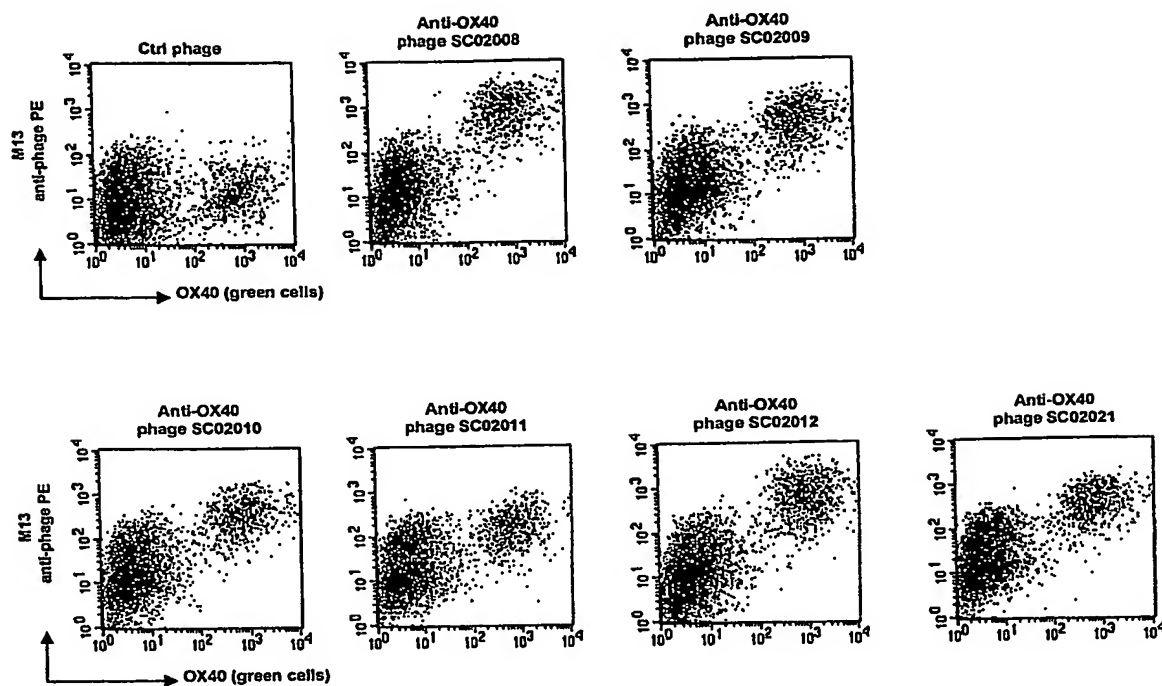


Figure 2



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Figure 3A

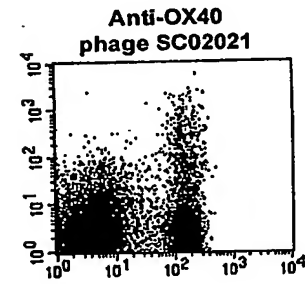
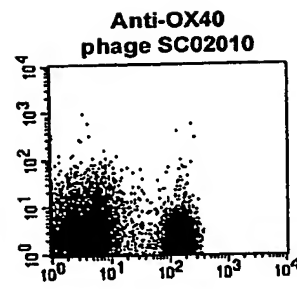
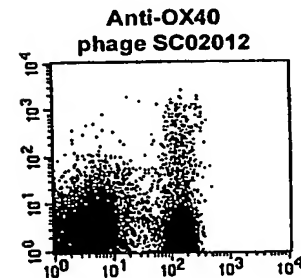
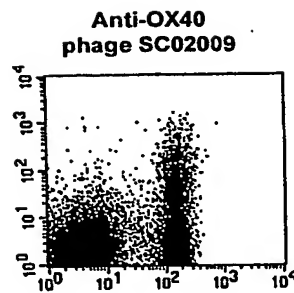
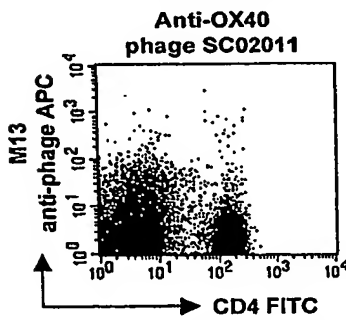
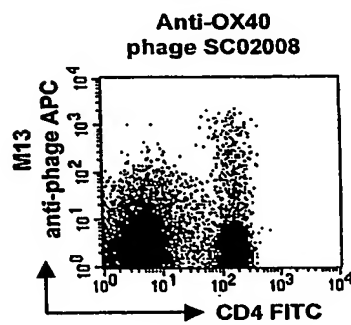
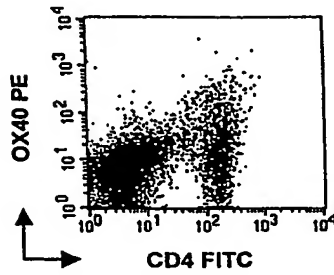
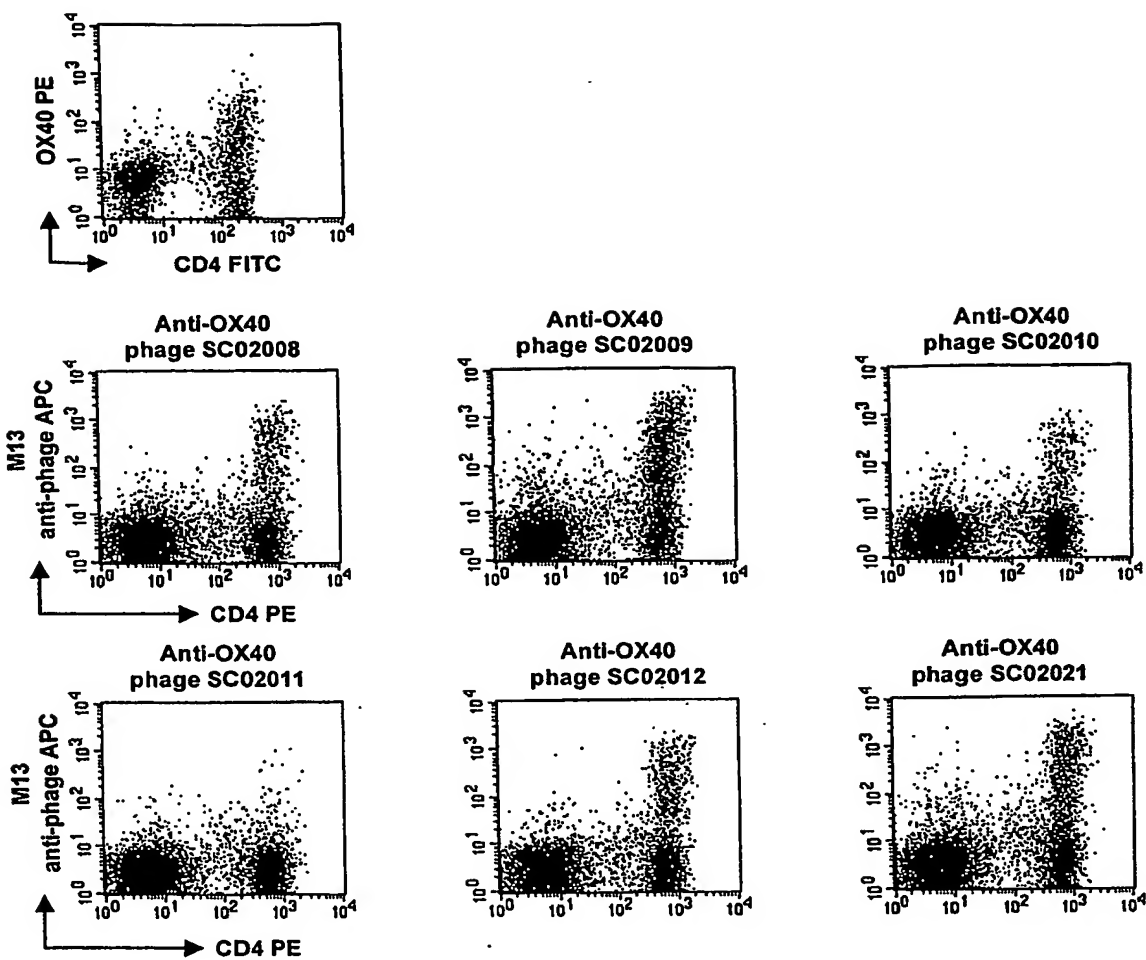


Figure 3B -



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Figure 3C -

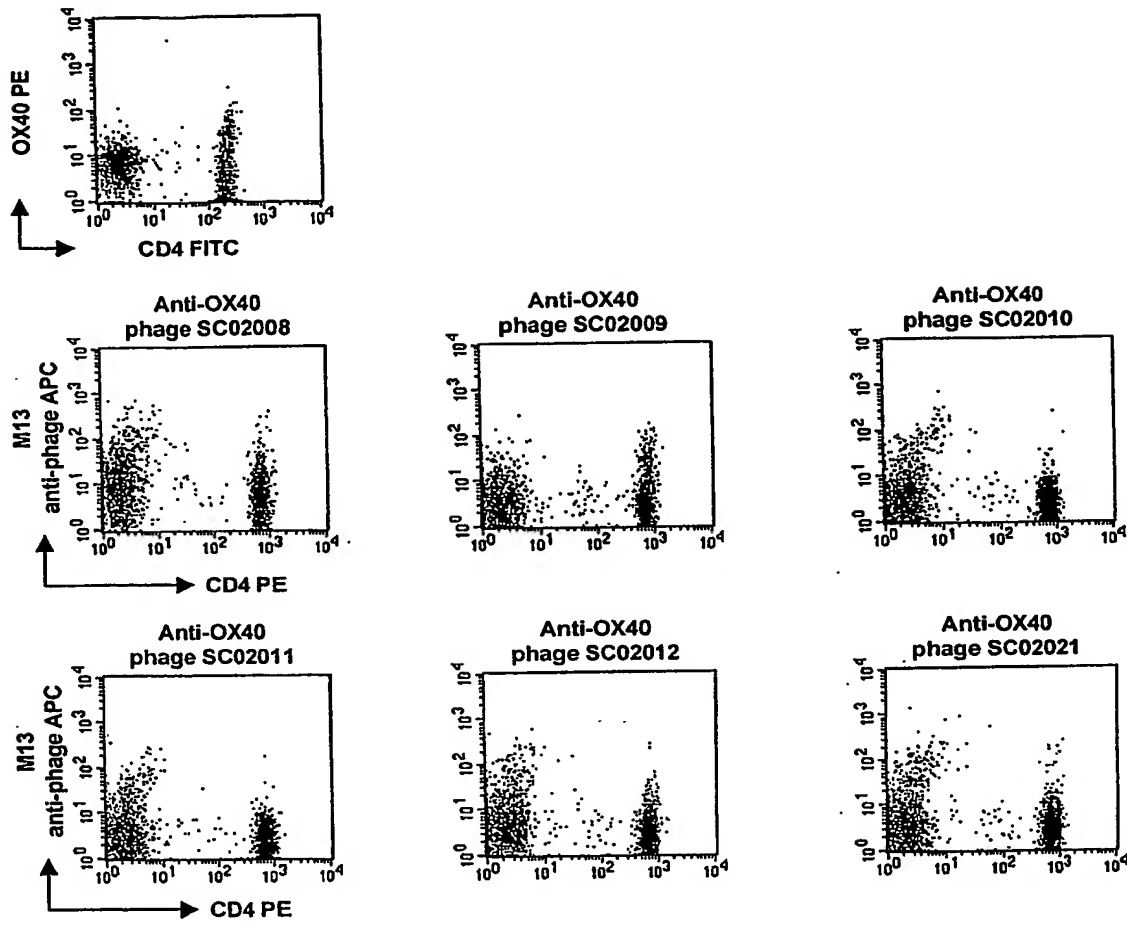
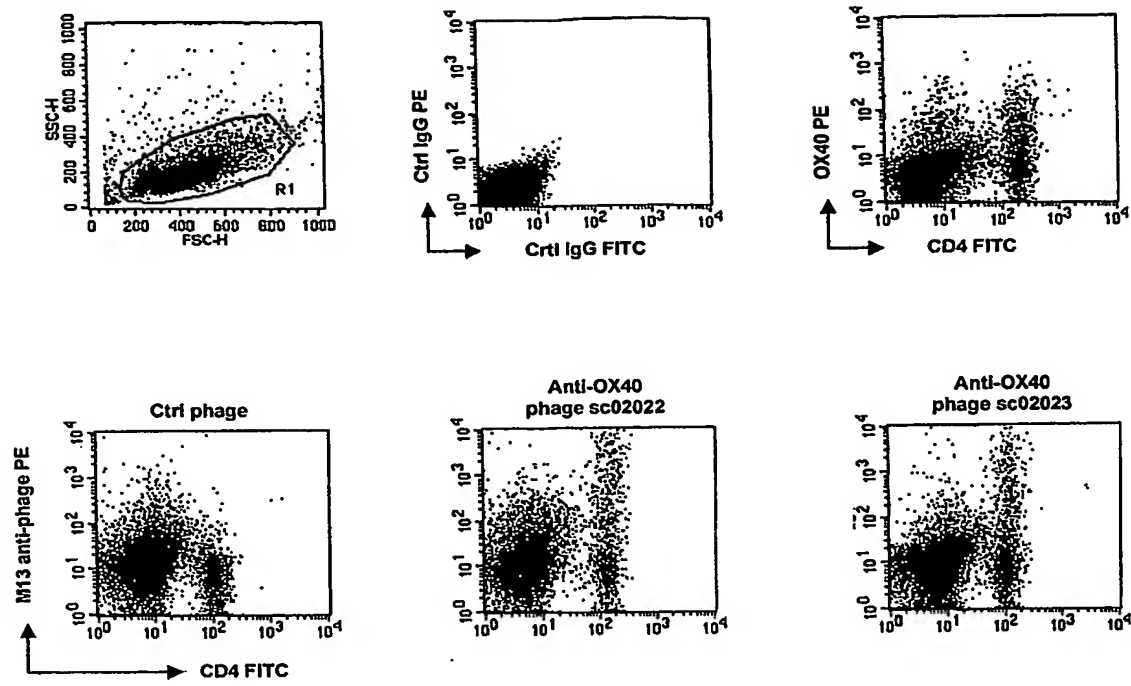


Figure 4A



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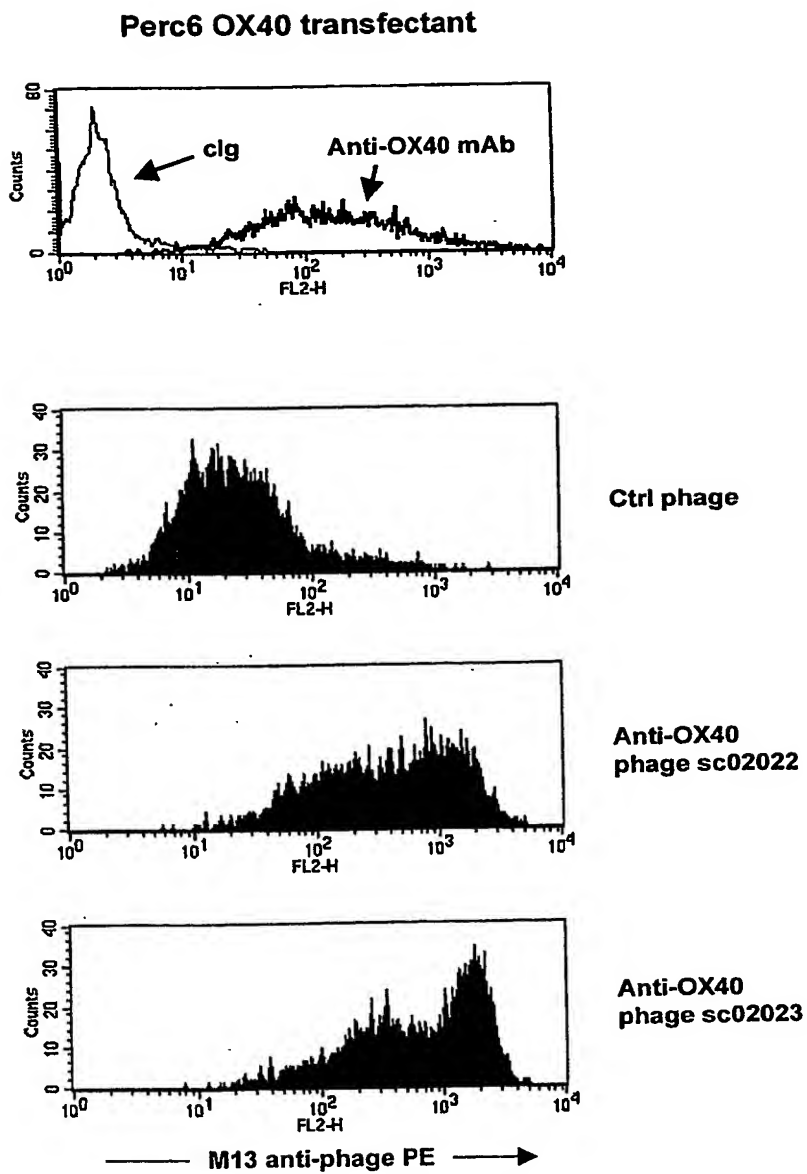
Figure 4B⁻

Figure 5 -

Anti-human OX40R scFv SC02008

NcoI

143 M A E V Q L V E S G G G L V Q P G G S L R
CCATGGCTGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTGGTCCAGCCTGGAGGGTCCCTGAG
214 L S C A A S G F T F S N Y T M N W V R Q A P G
ACTCTCCTGTGCAGCCTCTGGATTACCTTTAGCAACTACACGATGAACTGGGTCCGCCAGGCGCCCGGGA
285 K G L E W V S A I S G S G G S T Y Y A D S V K G
AGGGGCTGGAGTGGGTCTCAGCTATTAGTGGTAGTGGTGGTAGCACATACTACGCAGACTCCGTGAAGGGC
356 R F T I S R D N S K N T L Y L Q M N S L R A E D
CGGTTCAACATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAATGAACAGCCTGAGAGCCGAGGA
427 T A V Y Y C A K D R Y S Q V H Y A L D Y W G Q
CACGGCCGTGTATTACTGTGCCAAGACCGCTACTCCCAGGTGCACTACGCGTTGGATTACTGGGGCCAGG
498 G T L V T V L E G T G G S G G T G S G T G T S E
GCACCCCTGGTGACCGTGCTCGAGGGTACCGGAGGTTCCGGCGGAACCGGGTCTGGGACTGGTACGAGCGAG
569 L D I Q M T Q S P D S L P V T P G E P A S I S C
CTCGACATCCAGATGACGCAGTCTCCAGACTCACTGCCCCGTCACCCCTGGAGAGCCGGCCTCCATCTCCTG
640 R S S Q S L L H S N G Y N Y L D W Y L Q K A G
CAGGTCTAGTCAGAGCCTCCTGCATAGTAATGGATACAACATATTTGGATTGGTACCTGCAGAAGGCAGGGC
711 Q S P Q L L I Y L G S N R A S G V P D R F S G S
AGTCTCCACAGCTCCTGATCTATTTGGGTTCTAATCGGGCCTCCGGGGTCCCTGACAGGTTAGTGGCAGT
782 G S G T D F T L K I S R V E A E D V G V Y Y C Q
GGATCAGGCACAGATTTTAACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCCA
NotI

853 Q Y Y N H P T T F G Q G T K L E I K R A A
GCAGTACTACAACCCGACGACCTTCGGCCAGGGCACCAAACTGGAATCAAACGCGCGGCCGC

CDR3H: DRYSQVHYALDY

Figure 6

Anti-human OX40R scFv SC02009

NcoI
~~~~~

143                   M A E V Q L V E S G G G L  
                  CCATGGCTGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTG

214           V Q P G G S L R L S C A A S G F T F S G Y S M N  
              GTCCAGCCTGGGGGTCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTCAGCGGCTACTCTATGAA

285           W V R Q A P G K G L E W V G R T R N K A N S Y  
              CTGGGTCCGCCAGGCGCCCGGAAGGGGCTGGAGTGGGTGGCCGTACTAGAAACAAAGCTAACAGTTACA

356           T T E Y A A S V K G R F T I S R D D S K N S L Y  
              CCACAGAATACGCCGCTCTGTGAAAGGCAGATTCAACCATCTCAAGAGATGATTCAAAGAACTCACTGTAT

427           L Q M N S L R A E D T A V Y Y C A K D R Y V N T  
              CTGCCAATGAACAGTCTGAGAGCCGAGGACACAGCCGTGTATTACTGTGCCAAAGACCGCTACGTCAACAC

498           S N A F D Y W G Q G T L V T V L E G T G G S G  
              GTCGAACGCGTTCGATTACTGGGGCCAGGGCACCTGGTGACCGTGCTCGAGGGTACCGGAGGTTCCGGCG

569           G T G S G T G T S E L D I Q M T Q S P D S L P V  
              GAACCGGGTCTGGGACTGGTACGAGCGAGCTCGACATCCAGATGACACAGTCTCCAGACTCACTGCCCGTC

640           T P G E P A S I S C R S S Q S L L H S N G Y N Y  
              ACCCCTGGAGAGCCGGCCTCCATCTCCTGCAGATCTAGTCAGAGCCTCCTGCATAGTAATGGATACAATA

711           L D W Y L Q K P G Q S P Q L L I Y L G S N R A  
              TTTGGATTGGTACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTGGGTTCTAATCGGGCCT

782           S G V P D R F S G S G S G T D F T L K I S R V E  
              CCGGGGTCCCTGACAGGTTCAAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATCAGCAGAGTGGAG

853           A H H V G V Y Y C Q Q Y P L G P P T F G Q G T K  
              GCTCACCATGTTGGGGTTTATTACTGCCAGCAGTACCCGCTGGGCCCGCCACCTTCGGCCAGGGCACCAA

NotI  
~~~~~

924 L E I K R A A
 ACTGGAAATCAAACGCGCGGCCGC

CDR3H: DRYVNTSNAFDY

10/19

Figure 7

Anti-human OX40R scFv SC02010

72
143
214
285
356
427
498
569
640
711
782
853

ESGGGLIQPGGSLRLSCAASGFTF
AGTCTGGGGGAGGCTTGATCCAGCCTGGGGGGTCCCTGAGACTCTCCTGTGCAGCCTCTGGATTACCTTC
SGYPMNWVRQAPGKGLEWVAVISY
AGCGGCTACCTATGAAC TGGGTCCGCCAGGCGCCGGAAGGGGCTGGAGTGGGTGGCAGTTATATCATA
DGSNKYYADSVKGRFTISRDN SK
TGATGGAAGTAATAAATACTACG CAGACTCCGTGAAGGGCCGATT CACCATCTCCAGAGACAATCCAAGA
NTLYLQMNSLR AEDTAVYYCAR DM
ACACGCTGTATCTGCAAATGAACAGCCTGAGAGCTGAGGACACAGCCGTGTATTACTGTGCAAGAGACATG
SGFHEFDYWGQGTLVTVLEGGTGS
TCCGGCTTCCACGAGTTCGATTACTGGGGCCAGGGCACCCCTGGTGACCGTCTCGAGGGTACCGGAGGTTG
GGTGS GTGTSEL TQSPSSLASV
CGGCGGAACCGGGTCTGGGACTGGTACGAGCGAGCTCACCAGTCTCCATCTCCCTGTCTGCATCTGTAG
GDRVTITCRASQSIS SYLNWYQQK
GAGACAGAGTCACCATCACTTGCCGGGCAAGTCAGAGCATTAGCAGTACTTAAATTGGTATCAGCAGAAA
PGKAPKLLIYAASSSLQSGVPSRFS
CCAGGGAAAGCCCTAAGCTCCTGATCTATGCTGCATCCAGTTTGCAAAGTGGGGTCCCATCAAGGTTGAG
GSGSGTDFTLTIS SLQPEDFATY
TGGCAGTGGATCTGGGACAGATTTCACTCTCACCATCAGCAGTCTGCAACCTGAAGATTTTGCAACTTACT
Y C Q Q S Y S T P P T F G Q G T K V E I K R A A
ACTGTCAACAGAGTTACAGTACCCCTCCAACGTTTCGGCCAAGGGACCAAGGTGGAGATCAAACGTGCGGCC
GC

NcoI

M A E V Q L V
CCATGGCTGAGGTGCAGCTGGTGG

NotI

CDR3H: DMSGFHEFDY

11/19

Figure 8

Anti-human OX40R scFv SC02011

NcoI
~~~~~  
M A E V Q L V E S G G G V V Q P G R  
143 CCATGGCTGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCGTGGTCCAGCCTGGGAGGT  
S L R L S C A A S G F T F S D Y T M N W V R Q A  
214 CCCTGAGACTCTCTGTGCAGCCTCTGGATTACCTTCAGCGACTACACGATGAACTGGGTCCGCCAGGCG  
P G K G L E W V S S I S G G S T Y Y A D S R K G  
285 CCCGGGAAGGGGCTGGAGTGGGTCTCATCCATTAGTGGTGGTAGCACATACTACGCAGACTCCAGGAAGGG  
R F T I S R D N S K N T L Y L Q M N N L R A E  
356 CAGATTCACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTTCAAATGAACACCTGAGAGCTGAGG  
D T A V Y Y C A R D R Y F R Q Q N A F D Y W G Q  
427 ACACGGCCGTGTATTACTGTGCAAGAGACCGCTACTTCAGGCAGCAGAACGCGTTTCGATTACTGGGGCCAG  
G T L V T V L E G T G G S G G T G S G T G T S E  
498 GGCACCCCTGGTGACCGTGTCTCGAGGGTACCGGAGGTTCCGGCGGAACCGGGTCTGGGACTGGTACGAGCGA  
L D I Q M T Q S P V T L P V T P G E P A S I S  
569 GCTCGACATCCAGATGACTCAGTCTCCAGTCACCCCTGCCCCTCACCCCTGGAGAGCCGGCCTCCATCTCT  
C R S S Q S L L H S N G Y N Y L D W Y L Q K P G  
640 GCAGGTCTAGTCAGAGCCTCCTGCATAGTAATGGATACAATAATTTGGATTGGTACCTGCAGAAGCCAGGG  
Q S P Q L L I Y L G S N R A S G V P D R F S G S  
711 CAGTCTCCACAGCTCCTGTATCTATTGGGTTCTAATCGGGCCTCCGGGGTCCCTGACAGGTTCACTGGGCAG  
G S G T D F T L K I S R V E A E D V G V Y Y C  
782 TGGATCAGGCACAGATTTTACACTGAAAATCAGCAGAGTGGAGGCTGAGGATGTTGGGGTTTATTACTGCC  
NotI  
~~~~~  
Q Q Y L T A P P T F G Q G T K L E I K R A A
853 AGCAGTACCTCAGGCCCCCGCCACCTTCGGCCAGGGCACCAAACTGGAAATCAAACGCGCGGCCGC

CDR3H: DRYFRQQNAFDY

12/19

Figure 9

Anti-human OX40R scFv SC02012

72

NcoI
~~~~~  
M A E V Q L V E  
CCATGGCTGAAGTGCAGCTGGTGGA

214

285

356

427

498

569

640

711

782

NotI  
~~~~~  
K R A A
AACGCGCGGCCGC

853

S G G G L V K P G G S L R L S C A A S G F T F S
AAGCGGCGGCGGCTGGTGAAGCGGGTGGCAGCCTGCGCCTGAGCTGCGCCGCTAGCGGCTTCACCTTTA
N D S M N W M R Q A P G K G L E W V A N I N Q
GCAACGACTCGATGAACTGGATGCGCCAGGCCCGGGCAAAGCCCTCGAATGGGTGGCCAATATCAATCAG
D G N E K Y Y A D S V K G R F T I S R D N S K N
GATGGCAACGAAAAATATTACGCCGACTCTGTCAAAGGCCGCTTCACCATCAGTCGCGATAACTCCAAAAA
S L Y L Q M N S L R D E D T A L Y Y C A R A R
CTCCCTGTACCTGCAGATGAACAGCCTGCGCGACGAAGATACCGCCCTGTACTACTGCGCACGCGCCCGCG
A A G T I F D Y W G Q G T L V T V L E G T G G S
CCGCCGGCACCATCTTCGATTACTGGGGCCAGGGCACCCCTGGTGACCGTGCTCGAGGGTACCGGAGGTTCC
G G T G S G T G T S E L D I Q M T Q S P S S L S
GGCGGAACCGGGTCTGGGACTGGTACGAGCGAGCTCGATATCCAGATGACCCAGAGCCCGAGTTCCCTGAG
A S V G D R V T I T C R A S Q N V S N Y L T W
CGCCTCCGTGGGCGACCGCGTGACCATCACCTGCCGCGCCAGCCAGAACGTCAGCAACTACCTGACCTGGT
Y Q Q K P G K A G K L L I Y A A S S L Q S G V P
ACCAGCAGAAACCGGGCAAGGCTGGCAAACCTGCTGATTTACGCCGCCAGCAGCCTCCAAAGCGGCGTGCCG
S R F S G S G S G T D F T L T I S S L Q P E D F
TCTAGATTCACTGGCTCCGGCTCCGGAACCGATTTTACCCTGACCATCAGCAGCCTGCAGCCGGAAGATT
A T Y Y C Q Q S Y F N P A T F G Q G T K L E I
CGCTACCTACTATTGTACAGCAGTCCTACTTCAACCCGGCGACCTTCGGCCAGGGCACCAAACCTGGAATCA

CDR3H: ARAAGTIFDY

Figure 10

Anti-human OX40R scFv SC02021

NcoI
~~~~~

143 M A E V Q L V E S G G G L  
CCATGGCTGAGGTGCAGCTGGTGGAGTCTGGGGGAGGCTTG

214 V Q P R G S L R L S C A A S G F T F S S Y A M N  
GTACAGCCTAGGGGGTCCCTGAGACTCTCTGTGCAGCCTCTGGATTACCTTTAGCAGCTACCGGATGAA

285 W V R Q A P G K G L E W V A V I S Y D G S N K  
CTGGGTCCGCCAGGCGCCCGGAAGGGGCTGGAGTGGGTGGCAGTTATATCATATGATGGAAGCAATAAAT

356 Y Y A D S V K G R F T I S R D N S K N T L Y L Q  
ACTACGCACTCCGTGAAGGGCCGATTACCATCTCCAGAGACAATTCCAAGAACACGCTGTATCTGCAA

427 M N S L R A E D T A V Y Y C A K D R Y I T L P N  
ATGAACAGCCTGAGAGCTGAGGACACAGCCGTGTATTACTGTGCCAAAGACCGCTACATCACGTTGCCGAA

498 A L D Y W G Q G T L V T V L E G T G G S G G T  
CGCGTTGGATTACTGGGGCCAGGGCACCCTGGTGACCGTGCTCGAGGGTACCGGAGGTTCCGGCGGAACCG

569 G S G T G T S E L D I Q M T Q S P V S L P V T P  
GGTCTGGGACTGGTACGAGCGAGCTCGACATCCAGATGACCCAGTCTCCAGTCTCACTGCCCCTCACCCCT

640 G E P A S I S C R S S Q S L L H S N G Y N Y L D  
GGAGAGCCCGCTCCATCTCCTGCAGGTCTAGTCAGAGCCTCCTGCATAGTAATGGATACAACATTTTGA

711 W Y L Q K P G Q S P Q L L I Y L G S N R A S G  
TTGGTACCTGCAGAAGCCAGGGCAGTCTCCACAGCTCCTGATCTATTTGGGTTCTAATCGGGCCTCCGGGG

782 V P D R F S G S G S G T D F T L K I S R V E A E  
TCCCTGACAGGTTAGTGGCAGTGGATCAGGCACAGATTTTACACTGAAAATCAGCAGAGTGGAGGCTGAG

853 D V G V Y Y C Q Q Y K S N P P T F G Q G T K V E  
GATGTTGGGTTTATTACTGCCAGCAGTACAAGTGAACCCGCCCACCTTCGGCCAGGGCACCAAGTGA

NotI  
~~~~~

924 I K R A A
AATCAAACGCGCGGCCGC

CDR3H: DRYITLPNALDY

NcoI

72

143

214

285

356

427

498

569

640

711

782

M A E E V Q L V E S S G G G G G C
CATG GCCCGAGGATG CAGCTGGTGGAGTCTGGGGGGAGGC

L V H P G G S L R L S C A G G S G F T F S S Y A M
TTGTGATACATCCTGGGGGGTCCCTGAGACTCTCTGTG CAGGCTCTGGATTACCTTCAGTAGCTATGCTAT

H W V R Q A P G K G L E W V S A I G T G G G T
GCACTGGGTTGCCAGGCTCCAGGAAAAGGTCTGGAGTGGGTATCAGCTATTGGTACC GG TGGTGGCACAT

Y Y A D S V Q G R F T I S R D N A K N S L Y L Q
ACTATGCAGACTCCGTGCAGGGCCGATTACCATCTCCAGAGACAATGCCAAGAACTCCTTGATCTCTCAA

M N S L R A E D T A V Y Y C A R Y D E P L T I Y
ATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGATACGACGAGCCGCTGACGATTTA

W F D S W G Q G T L V T V S S G G G G S G G G
CTGGTTTGACTCTCTGGGGCCAAGGTACCTTGGTCAACGCTCTCGAGTGGTGGAGGCGGTT CAGCGGAGGTG

G S G G G G S E I E L T Q S P A T L S L S P G E
GCTCTGGCGGTGGCGGATCGGAAATTGAGCTCACACAGTCTCCAGCCACCCTGTCTTTGTCTCCAGGGGAA

R A T L S C R A S Q S V S S Y L A W Y Q Q K P G
AGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTTAGCAGTACTTAGCCTGGTACCAACAGAAACCTGG

Q A P R L L I Y D A S N R A T G I P A R F S G
CCAGGCTCC CAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCC CAGCCAGGTT CAGTGGCA

S G S G T D F T L T I S S L E P E D F A V Y Y C
GTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTG CAGTTTATTACTGT

NotI
~~~~~

Q Q R S N W P P A F G G G T K V E I K R A A  
CAGCAGCGTAGCAACTGGCCTCCGGCTTTTCGGCGGAGGGACCAAGGTGGAGATCAAACGTCGGGCGGC

CDR3H: YDEPLTIYWFDS

## Figure 12

## Anti-human OX40R scFv SC02023

NcoI  
~~~~~  
M A E V Q L V E

72 CCATGGCCGAGGTGCAGCTGGTGGAG

143 S G G G L V H P G G S L R L S C A G S G F T F S
TCTGGGGGAGGCTTGGTACATCCTGGGGGTCCCTGAGACTCTCCTGTGCAGGCTCTGGATTACCTTCAG

214 S Y A M H W V R Q A P G K G L E W V S A I G T
TAGCTATGCTATGCACTGGGTTCCGAGGCTCCAGGAAAAGGTCTGGAGTGGGTATCAGCTATTGGTACTG

285 G G G T Y Y A D S V M G R F T I S R D N S K N T
GTGGTGGCACATACTATGCAGACTCCGTGATGGGCCGGTTCACCATCTCCAGAGACAATTCCAAGAACACG

356 L Y L Q M N S L R A E D T A V Y Y C A R Y D N V
CTGTATCTGCAATGAACAGCCTGAGAGCCGAGGACACGGCCGTGTATTACTGTGCAAGATACGACAATGT

427 M G L Y W F D Y W G Q G T L V T V S S G G G G
GATGGGTCTTTACTGGTTTGACTACTGGGGCCAAGGTACCCTGGTCACCGTCTCGAGTGGTGGAGGCGGTT

498 S G G G G S G G G G S E I E L T Q S P A T L S L
CAGGCGGAGGTGGCTCTGGCGGTGGCGGATCGGAAATTGAGCTCACACAGTCTCCAGCCACCCTGTCTTTG

569 S P G E R A T L S C R A S Q S V S S Y L A W Y Q
TCTCCAGGGGAAAGAGCCACCCTCTCCTGCAGGGCCAGTCAGAGTGTAGCAGCTACTTAGCCTGGTACCA

640 Q K P G Q A P R L L I Y D A S N R A T G I P A
ACAGAAACCTGGCCAGGCTCCAGGCTCCTCATCTATGATGCATCCAACAGGGCCACTGGCATCCCAGCCA

711 R F S G S G S G T D F T L T I S S L E P E D F A
GGTTCAGTGGCAGTGGGTCTGGGACAGACTTCACTCTCACCATCAGCAGCCTAGAGCCTGAAGATTTTGCA

782 V Y Y C Q Q R S N W P P A F G G G T K V E I K R
GTTTATTACTGTGAGCAGCGTAGCAACTGGCCTCCGGCTTTCGGCGGAGGGACCAAGGTGGAGATCAAACG

NotI
~~~~~  
A A

853 TCGGCGCGC

CDR3H: YDNVMGLYWFDY

Figure 13A

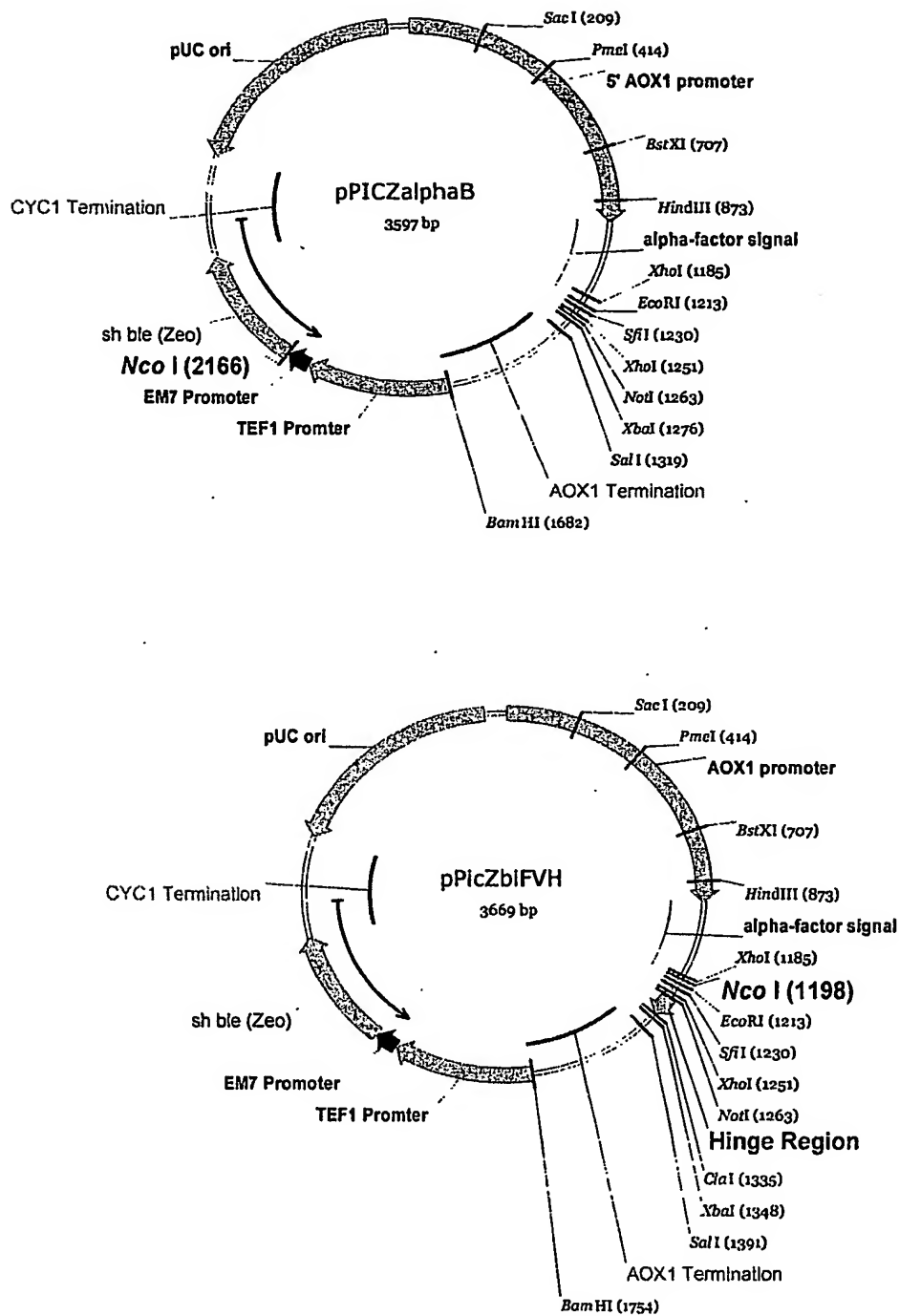


Figure 13B

5' Cloning site of pPicZαB

XhoI                      KEK2                      EcoRI                      SfiI  
 ~~~~~                      Cleavage                      ~~~~~                      ~~~~~  
 site
 ↓
 S L E K R E A E A A G I H V A Q P A
 1151 TCTCTCGAGA AAAGAGAGGC TGAAGCTGCA GGAATTCACG TGGCCCAGCC GGCCG
 AGAGAGCTCT TTTCTCTCCG ACTTCGACGT CCTTAAGTGC ACCGGGTCGG
 CCGGC

5' Cloning site of pPicZFH

XhoI KEK2 NcoI EcoRI SfiI
 ~~~~~                      Cleavage                      ~~~~~                      ~~~~~  
                                          site  
                                          ↓  
 S L E K R A M E A A G I H V A Q P A  
 1151 TCTCTCGAGA AAAGAGCCATGGAAGCTGCA GGAATTCACG TGGCCCAGCC GGCCG  
       AGAGAGCTCT TTTCTCGGTACCTTCGACGT CCTTAAGTGC ACCGGGTCGG CCGGC

synthetic hinge fragment

←                      Flexible upper                      Cysteine residues  
                                          hinge region                      available for  
                                                               disulphide bonding  
                                                               ↙                      ↘  
 NotI                      ~~~~~  
 ~~~~~  
 1 A A A P K P S T P P G S S C P P C
 GCGGCCGCGC CAAAGCCAAG TACCCACCA GGTCTTCAT GTCCACCATG
 CGCCGGCCGCGC CCGGTTT ATGGGGTGGT CCAAGAAGTA CAGGTGGTAC
 Short linker
                                          ~~~~~  
                                          ClaI                      XbaI  
                                          ~~~~~                      ~~~~~  
 51 P G S G G A P I D S G F L
 TCCAGGCTCT GCGGTGCGC CAATCGATAG CGGCTTTCTA GA
 AGGTCCGAGA CCGCCACGCG GTTAGCTATC GCCGAAAGAT CT

18/19

Figure 14A

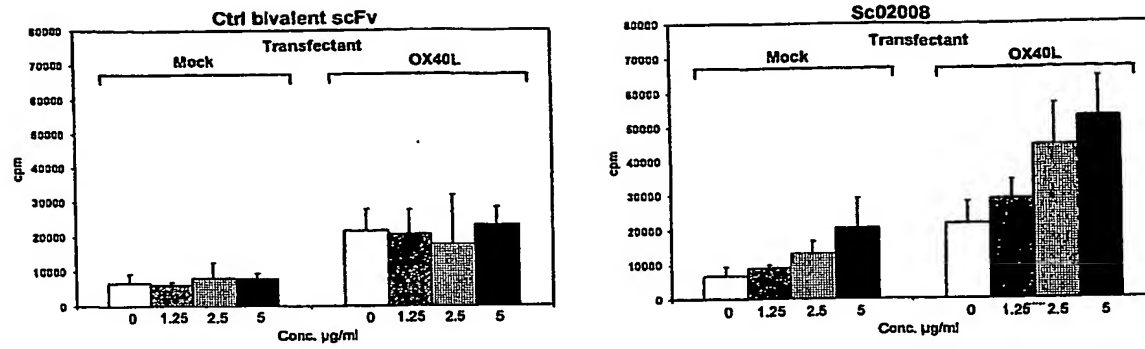


Figure 14B

